Research Article

Evaluation of Anticancer Potential of Biogenic Copper Oxide Nanoparticles (CuO NPs) against Breast Cancer

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The present study evaluated the anticancer potential of copper oxide nanoparticles (CuO NPs) synthesized from pumpkin seed extract in human breast cancer cell line (MDA-MB-231) using a battery of tests such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, morphological alteration, reactive oxygen species (ROS) generation, and changes in mitochondrial membrane potential (MMP). The biogenic CuO NPs showed a dose-dependent decline in cell viability with 50% inhibitory concentration (IC50) at 20 μg/ml. Treatment with an IC50 dose of CuO NPs resulted in considerable morphology changes, such as shrinkage, detachment, membrane blebbing, and deformed shape in MDA-MB-231 cells. We also observed a significant dose-dependent increase in ROS production and MMP modulation due to CuO NP treatment. Overall, CuO NPs showed significant anticancer potential in the breast cancer cell line. However, further validation of our data is required in ex vivo and in vivo models before this nanoformulation could be exploited for the treatment/management of human breast cancer.

1. Introduction

Cancers are considered one of the foremost reasons of mortality, where 1.9 million new cases and around 609,360 cancer deaths are expected in the United States by the end of 2022 [1]. Breast cancer (BC) is the most common cancer in women globally and the primary cause of cancer-related deaths in women, with the second-highest incidence rate (11.6%) among all cancers, necessitating the development of effective therapies [2, 3]. Currently, available chemotherapeutics for BC are costly, have frightening side effects, and could also lead to resistant cells [4–6]. Traditional therapies can reduce aggressive BC to moderately invasive BC; however, most invasive kinds have no effective treatment till now. Therefore, the urgent need is to find an effective, bio-compatible, and cost-effective therapeutic agent for BC especially invasive one, that has few or no adverse effects [7].

In this regard, cancer nanomedicine has taken a significant stride in the last few decades and improved the therapeutic index of cancer drugs [8, 9]. Nanotechnology has extensive application in biomedical sciences, particularly cancer therapeutics [10]. The advantages of nanomaterials are their large surface area and small particle size, making them excellent for synthesizing pharmaceutical formulations [11–13]. Metal oxide nanoparticles have recently come up as a promising research area due to their vast range of applications [9, 14]. Copper oxide nanoparticles (CuO NPs) have been widely studied nanoformulation owing to their intriguing physical, biochemical, and pharmacological features [9, 15]. Cu-based products have been permitted for human
use by the United States Environmental Protection Agency (USEPA) since February 2008 [7]. These nanoparticles are widely explored because they are essential trace element and have significant roles in metabolism and physiological processes [16, 17]. Different tumor cells, such as lung adenocarcinoma (A549), leukemia monocytic cells (THP-1), and colon cancer (HCT-116), have exhibited substantial toxicity to these nanoparticles [7, 9, 18].

Plant seeds are considered a significant source of biogenic nanoparticles production [19–21]. Among plants, pumpkin is a popular vegetable found in many foods such as bonbons, comestibles, and rice cakes and has shown several benefits [22]. The diverse bioactive compounds such as carotenoids, polysaccharides, para-aminobenzoic acid, fixed oil, sterol, protein, and peptides in pumpkins make them suitable against various cancer [23, 24]. Previous studies have shown the significant anticancer potential of pumpkins against gastric, breast, lung, colon, and prostate cancer [25, 26]. Biogenic nanoparticles have gained considerable attention lately because of their low-cost, eco-friendly nature, reliability, and relative safety [27, 28].

In our earlier study, we successfully synthesized and characterized biogenic CuO NPs from pumpkins seed extract using a green, environmental friendly, and nontoxic approach [9]. The biosynthesized CuO NPs showed significant anticancer against HCT-116 cell lines. The current study is a continuation of our previous work, and it aims to analyze the anticancer potential of CuO NPs in breast cancer cell lines (MDA-MB-231) in order to compare its efficacy. Based on the findings, we intend to expand our research to the most responsive cancer model.

2. Materials and Methods

All the chemicals utilized in this investigation were acquired commercially from companies like Merck, Sigma, and others. Streptomycin, penicillin, phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH), trypsin-EDTA, acridine orange, and ethidium bromide were obtained from the companies mentioned above. All the other chemicals were purchased locally and were of analytical grade.

2.1. Synthesis and Characterization of CuO NPs. In our previous study, we described the synthesis and characterization of CuO NPs from pumpkin seed extract in detail [9]. Briefly, the pumpkin seed extract was prepared and subsequently added in 3 mM of Cu(OAc)₂ solution with continuous stirring. The addition of NaOH to the extract solution resulted in the formation of CuO NPs. CuO NPs were characterized using a variety of analytical techniques, including UV-vis absorption spectroscopy, Fourier transform infrared spectrum analysis (FTIR), X-ray diffraction (XRD), energy dispersive X-ray analysis (EDX), scanning electron microscopy (SEM), and transform electron microscopy (TEM). These techniques confirmed the biogenic synthesis of CuO NPs in the 20 nm range.

2.2. Cell Culture Maintenance. The National Centre for Cell Sciences (NCCS) in Pune, India, provided the MDA-MB-231 breast cancer cell line. The cell line was grown in DMEM media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 g/ml) at 37°C in 5% CO₂ incubator.

2.3. Cytotoxicity (MTT) Assay. To measure cytotoxicity, MDA-MB-231 cells were treated with different doses of CuO NPs (5–35 μg/ml) in 96-well plate for 24 hours. After treatment, each well was added with 10 μl of MTT, followed by 2 hours of incubation at 37°C. The purple precipitated formazan was dissolved by adding 100 μl of DMSO, and the absorbance was measured at 540 nm using a multiwell plate reader [9]. The cytotoxicity of CuO NPs was calculated by comparing the percentage of treated cells to control cells.

Inhibitory of cell proliferation (%) = (Mean absorbance of the control – Mean absorbance of the sample)/(Mean absorbance of the control) × 100.

The sample CuO NP dose-responsive curve was used to calculate the 50% inhibitory concentration (IC50).

2.4. Induction of Apoptosis Using Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining Technique. Microscopic fluorescence assessment of apoptotic induction was performed using the approach described by Baskic et al. [29]. Before being examined under a fluorescence microscope, the treated cells were rinsed in cold PBS and stained with AO/EB (1:1 ratio; 100 μg/ml) for 5 minutes. The number of cells undergoing apoptosis was computed as a fraction of the total number of cells (40× magnification).

2.5. Measurement of Reactive Oxygen Species (ROS). Intracellular ROS generation was detected using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) [30]. After washing the treated cells with PBS, it was exposed with 25 μM DCFH-DA for 30 minutes at 37°C as we reported previously [27]. The fluorescence was measured every 5 minutes for up to 30 minutes using a spectrofluorometer (excitation 485 nm and emission 535 nm) (Shimadzu, Columbia, USA). A mean slope/min was used to calculate the increase in ROS production, which was then normalized to the unexposed control cells.

2.6. Measurement of Mitochondrial Membrane Potential (MMP). The established approach by Bhosle et al. [31] was
used to measure the modulation in mitochondrial membrane potential. The Rh-123 dye was used to stain the treated cells and was incubated for 15 minutes. The cells were fixed after being washed twice with PBS, and the fluorescence intensity was measured at 535 nm.

3. Results and Discussion

3.1. Cytotoxicity Assay and Morphological Alterations. A concentration-dependent rise in cytotoxicity was recorded in the MDA-MB-231 cell line in response to the treatment of CuO NPs. The IC50 concentration was found to be 20 μg/ml. The cell viability percentage was reduced to 24% at the highest tested dose, i.e., 35 μg/ml (Figure 1). The cytotoxicity of CuO NPs synthesized from various biological sources has previously been reported in several cancers cell lines, such as HepG2, Amj 13, MCF-7, MDA-MB-231, A549, and HCT-116 [9, 32–36]. The characteristics of NPs, such as nanoparticle size, surface charge, and functional groups determine the therapeutic potential of NPs [11]. A recent study highlighted the better pharmaceutical and biomedical capacity of CuO NPs with smaller NPs sizes [37]. The advantage of our synthesized CuO NPs is the lower particle size (20 nm) compared to earlier reported ones [37]. The smaller size of CuO NPs could results in extensive tissue distribution, deeper penetration inside specific tissues, better cellular uptake, and increased toxic effects to the cancer cells [37–39]. Earlier studies also reported higher IC50 of CuO NPs in human breast cancer cell lines compared with our biogenic CuO NPs, i.e., 20 μg/ml indicating its better efficacy [34, 35]. The smaller size and varied surface characteristics of CuO NPs could explain the lower IC50 value of our nanoformulation.

In addition, CuO NP treatment caused morphological changes in MDA-MB-231 cells, such as shrinkage, detachment, membrane blebbing, and distorted shape. On the other hand, control cells showed typical intact cell morphology (Figure 2).

3.2. Induction of Apoptosis in Response of CuO NP Treatment. Living cells showed green fluorescence and had normal nuclear appearance. However, a significant induction of apoptosis was observed at 20 μg/ml CuO NP concentration. Figure 3 depicts a fragmented nucleus showing yellow fluorescence with condensed chromatin, indicating early apoptotic cells. However, the orange fluorescence with chromatin condensation or fragmentation (uniformly red/orange-stained cell nuclei) indicate late apoptotic cells. In addition, we also quantitatively measured the percentage of apoptotic cells, which showed 57% apoptotic cells at 25 μg/ml of CuO NP treatment (Figure 4).

Apoptosis is considered a significant anticancer mechanism that involves the activation of a sequence of molecular events culminating into cell death with cellular,
morphological, and biochemical changes [40]. It is well-known that excessive generation of ROS/RNS, oxidative stress, and cancer cell Sub G1 arrest is connected to DNA damage and apoptosis/necrosis [41, 42]. Our results agree with earlier studies that observed induction of apoptosis as a result of green synthesized nanoparticles [40, 43]. Of late, endoplasmic reticulum stress-mediated induction of apoptosis has also been reported in response to CuO NP treatment in Wistar rats [44]. Other apoptosis-promoting pathways have also been identified in response to green synthesized copper nanoparticles in Hep-2 and MCF-7 cells, that include upregulation of tumor suppressor genes (p53, Bax, caspase-3, and caspase-9) and downregulation of oncogenes (Ras and Myc) [45, 46].


Control cells (dull green fluorescence) and CuO NP treated cells showed bright DCF stained green fluorescence indicating production of ROS (Figure 5). The ROS generation was also quantified by estimating fluorescence intensity (Au) in breast cancer cells (Figure 6), showing increased formation of ROS in a dose-dependent manner. Our findings support previous research that identified enhanced ROS generation as the key cytotoxic mechanism of green synthesized CuO NPs [41, 47]. In addition, our study also demonstrated comparatively higher ROS production at lesser dose of CuO NP. Increased generation of ROS is important for cell apoptosis regulation [35].
3.4. Effects of CuO NPs on the Mitochondrial Membrane Potential (MMP) in MDA-MB-231 Cells. We observed a gradual decrease in green fluorescence with an increasing concentration of CuO NPs, indicating a dose-dependent decline in MMP. The fluorescent image at 40× magnification shows rhodamine accumulation in control cells and its absence in treated cells (Figure 7). Modulation in MMP was also quantified by estimating fluorescence intensity (Au) in MDA-MB-231 cells indicating a notable change in MMP in response to CuO NP treatment (Figure 8). Green synthesized CuO NPs from black bean extract have also affected the mitochondrial structure and modulated membrane potential in HeLa cells [36]. Induction of apoptosis increased formation of ROS/NO, loss of MMP, etc. has been suggested as possible mechanisms of action of green synthesized NPs in the scientific literature [43, 48, 49]. Our biogenic synthesized NPs are also adopting the same mechanism of action for their anticancer effects.

4. Conclusion

The current study exploited an environmentally safe and biogenic approach for synthesizing CuO NPs from pumpkin seed extract. Our findings suggest a robust anticancer potential of CuO NPs, indicating induction of apoptosis, increased formation of ROS, and loss of MMP as possible mechanism of action. We advocate validating our in vitro results in ex vivo and in vivo models, given the considerable benefits of these NPs. Adequate replication of our findings could lead to the utilization of these biosynthesized CuO NPs in pharmacological, clinical, and biotechnological domains. Understanding the specific mechanism of action of these NPs could also provide a better insight into their application in different fields.

Data Availability

Data will be available upon genuine request to corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors’ Contributions

ST and AAM conceived, guidance, funding, and made first/final draft. MS, MK, and TAZ performed biological experiments. NRJ and SW did the statistical analysis, presented the results in a scientific manner, and also made the article’s first draft.

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